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The subject of this paper was suggested by Prof. Andrew Boss, while the details as to methods of procedure, helpful suggestions, and criticisms were given by Dr. R. B. Harvey. To these, thanks are due for providing ways and means for carrying out this work. To the section of plant physiology, Division of Plant Pathology and Botany, thanks are due for providing the major facilities needful for the completion of the work; to the Division of Biochemistry, for the use of apparatus; and to Dr. R. A. Gortner and Dr. J. J. Willaman for helpful suggestions. To Prof. A. C. Arny, who provided means and materials for the work, especially during the early investigational period; and to Mr. Esten Hendricks for his competent assistance in the carbohydrate and amino-nitrogen determinations, thanks are due.

# WINTER HARDINESS IN ALFALFA VARIETIES[1]

By Ferdinand H. Steinmetz

## INTRODUCTION

The importance of alfalfa as a forage crop is generally known. Likewise, the uncertainty of successful overwintering of the crop is known to be one of the greatest hazards of the alfalfa growers. This is particularly true in the northern part of the United States and in Canada, where the crop is of great importance to the livestock industry. Winter-hardy forms of alfalfa have been introduced from Europe, where they were found growing under climatic conditions similar to those in northern United States. Additional hardy forms have been produced by selection, while the production of hardy strains by hybridization is at present being carried on by several plant breeders. Types of root systems (50)[2] and the degree of dormancy (7) have been correlated with winter-hardy forms. However, in the final analysis, the isolation of winter-hardy strains has been dependent upon natural selection over several years. It is well known that so-called test winters occur occasionally—at intervals of ten or more years. Winter hardiness in winter wheat has been studied recently by Newton (32, 34), who used various methods and devices to measure the relative hardiness of wheat varieties. It was the purpose of this experimental work to employ available methods and apply them to two commonly recognized varieties of alfalfa—Grimm and Kansas grown common—with the hope that some light might be thrown on the basic differences between them.

## WINTER KILLING OF SUBTERRANEAN PLANT PARTS

The work of previous investigators has been reviewed by Abbe (1), Blackman (5), Chandler (11), Rosa (38), Wiegand (48), and Newton (32). Since a large part of the literature of winter hardiness and winter killing is concerned principally with above-ground parts, a brief review only of the overwintering of subterranean parts and herbaceous plants will be given.

According to Goeppert (17), Theophrastus recommended covering the vine with soil and Cato recommended covering plants with straw to prevent winter killing. Goeppert (18) records temperature at Ustjanks, Siberia (70° 55″ N. Lat.), in which he gives the average temperature for the coldest month (January) to be —50° C. He also

[1] Also submitted to the Faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the Degree of Doctor of Philosophy, June, 1926.

[2] Figures in parentheses refer to Literature Cited, page 31.

records the temperature at the surface of the soil under 4 inches of snow for the period February 4 to 16, inclusive. In addition, he determined the depth to which the soil was frozen and its temperature 2 inches below the surface. To illustrate, on February 5 at 6 a.m. the temperature of the atmosphere was —21.5° C. and at 7 a.m. the snow temperature at the surface of the soil was —6.2° C., while the soil temperature 2 inches below the surface for the same day was —1.2° C. He recognized that the roots of trees and herbaceous perennials were protected from low temperatures, while the tops of trees and certain forms of cryptogamic plant life endured the low temperature. Carrick (10) has reviewed the horticultural literature relative to root injury ascribable to low temperature. He also presents data on the differences in hardiness of the roots of certain horticultural stocks. Wahlen (46) investigated various native and cultivated herbaceous legumes grown at different altitudes in Switzerland, and found that they showed no autonomous winter rest period. He further found that practically no variation in root contraction existed. By the use of microchemical methods he determined the type of reserve materials present in the roots during the winter. In addition he measured the relative amount of carbohydrate reserve material in the roots by the use of the specific gravity method. He further compared the specific gravity of roots from plants which had not been cut during the summer with that from plants which had been cut twice during the growing season. In general the differences found were slight. While cutting stimulated the use of root reserves, it was observed that they were readily replaced during active growth.

Zacharowa (51) very recently determined the effect of low temperatures upon the root tissues of seedlings of rye, wheat, corn, peas, and buckwheat, which were grown in an incubator at 22.0° C. Preliminary experiments brought forth the commonly stated fact that young or meristematic tissues are more resistant to freezing than older tissues. She found that the root tips were more resistant than any other root tissue under observation. She further reports that the cortex and the root hairs are least resistant; the meristem is most resistant; and the central cylinder takes an intermediate place. In addition, cytological and microchemical studies revealed that the meristem was the richest in protein and that carbohydrates were practically absent. The less hardy tissues showed appreciable amounts of carboydrates as reducing sugars. Salts which Maximow (29) considered of importance as protective substances were not found in the root tip. The least resistant root was buckwheat. It was also the most acid in reaction. Further study showed that the least hardy tissues of a root were also the most acid. It was found that the highly resistant meristematic

region of the root tip was alkaline in reaction. As further experimental material in a study of the effect of alkali and acid upon tissue, red cabbage was used. It was found that acid hastened the killing of the tissue, while alkali retarded it. As a result this author refutes the theory that sugars and salts are protective, but defends the theory of acid precipitation of cell proteins as a cause of death from freezing. Further evidence of the resistance of young tissue is found in Winkler's report (49). He points out in an extended list of plants that the young leaf-bud tissue is the most frost resistant part of young shoots.

Sylvén (44) reports an experiment in which hardy and non-hardy varieties of alfalfa were tested at Svalöf. He found Grimm to be one of the most hardy varieties. Another phase of his article deals with the time of cutting in autumn in relation to overwintering. He does not emphasize root reserves, but does differentiate between early and late fall cutting. He believes that early fall cutting forces the inactive buds into growth but does not provide enough time for new buds to reach a stage of development capable of overwintering. He observed that very late cutting (after hard frosts) did not result in increased winter injury.

Unpublished data by G. Nilsson-Leissner[3] showed that by the refrigeration method he was able to differentiate between the hardy and non-hardy strains of alfalfa. In addition, he reports that reducing sugars and total nitrogen determinations did not show significant varietal differences.

## MATERIALS AND METHODS

The experimental material consisted of roots of two physiologically different varieties of alfalfa. Grimm, a strain hardy under Minnesota conditions, has been described by Brand (6, 8), while Kansas grown common is a non-hardy form which has been grown for many years under conditions prevailing in the state of Kansas. All material used for physical and chemical data was grown in field plots under normal conditions. The seed was sown in May, 1922, without a nurse crop; and the experimental period, for the principal part, included the two winters between the fall of 1922 and the summer of 1924. Seedling plants which were used in studying dormancy and the killing points under controlled conditions, were grown in 6-inch flower pots embedded in the field. The roots for the expression of the cell sap, dry weight determinations, and chemical samples were dug from similar areas of contiguous plots. The tops were removed below the crown and the roots washed in tap water. Later the surface water was removed with

[3] Communication from Mr. Nilsson-Leissner, of Svalöf.

a towel, and an electric fan was used to remove all apparent surface moisture. During the winter, large lumps of frozen soil which separated at the plow sole approximately 7 inches deep were broken out with pickaxes. The frozen lumps were allowed to thaw over night in a warm room, after which the roots were removed as previously stated. Part of the soil was usually still frozen at the time the roots were removed.

The clean roots were then cut into pieces half an inch to an inch in length. After thoro mixing, duplicate samples for dry weight determinations were taken, and dried in an oven to constant weight. In addition, duplicate 50-gram samples for chemical analysis were dropped into fruit jars containing boiling alcohol and 1 gram of pure calcium carbonate. Enough 95 per cent alcohol was added to leave the final alcoholic concentration at 80 per cent [Spoehr (43), Davis, Daish, and Sawyer (13)]. The sample jars were then tightly sealed and stored. The remaining portion was put into a small cloth bag and frozen by the use of liquid $CO_2$ until the pieces were hard. This material was then ground with a food chopper, placed in closed containers, and kept in a cold place or put on ice. The freshly ground material was expressed in a large press operated by a hand screw which allowed the application of heavy pressure, or in a hydraulic press under a maximum pressure of 400 kilograms per square centimeter. The sap was then centrifuged for 10 minutes at a speed of approximately 2000 r.p.m., using an axis with a radius of 8 inches. A homogeneous liquid was thus prepared. The total solids were determined directly by the use of a standardized refractometer with a sugar scale attached, as was done by Gortner and Hoffman (21). The freezing point depression was determined with the Beckmann apparatus or a Heidenhain cryoscope with a range of $-7.5°$ C.

The depression of the freezing point of the root tissue was determined by the thermo-electric method, first proposed by D'Arsonval (4) and later improved by Dixon and Atkins (14); and used by several European and American investigators (2, 23, 37). White (47) has shown its accuracy. The arrangement of apparatus illustrated by Harvey (24) was used. By this method duplicates on the same root frequently varied from 0.3 to 0.5° C. Miss Payne (36) used this type of apparatus in studying the freezing point of various insect larvae. The actual freezing point depressions reported here represent the average of from six to twenty readings taken on from two to ten roots. The roots used were brought in from the field and kept on ice until determined. In no case were they out of the soil more than four hours. By this method the undercooling rarely was greater than $-2.0°$ C., and usually less than $-1.5°$ C. Corrections were made for

undercooling by the use of the formula given by Harris and Gortner (22). Since the completion of this experimental work, an investigation by Zacharowa (51) has been called to the writer's attention. She used the thermo-electric method to determine the freezing points of seedling roots of rye, wheat, corn, peas, and buckwheat. In order to protect the tender roots from desiccation she suspended them in small vials.

## RESULTS—PHYSICAL DETERMINATIONS

### DORMANCY AND REST PERIOD

Throughout the following discussion the term "rest period" will be considered as an autonomous condition in a plant during which it shows no growth activities and from which it will not spring into active growth when provided with favorable conditions; while "dormancy" is an apparently inactive condition forced upon plants by external conditions unfavorable to growth. In this paper the dormancy of alfalfa is considered as being brought on by low temperatures prevailing during winter.

Wahlen (47) found that *Medicago sativa* L., *Trifolium pratense* L., *Onobrychis viciaefolia* Scop. and *Anthyllis vulneraria* L. had no rest period. To obtain information on the relative dormancy of the two varieties under study, frozen lumps containing the roots were brought in from the field on January 16, 1923, and again on January 9, 1924, and placed in the greenhouse. Definite unfolding of young leaves was apparent in both varieties after 3 days. Potted plants were brought in each month throughout the two winters and used as checks on freezing experiments. Some measurements of the new growths were made, but the data do not indicate any difference in the rate of growth of the two varieties. Fluctuations in the rate of growth occurred in both varieties. These fluctuations apparently give an indication of the vigor of the individual plants. All observations under controlled conditions and in the field during early spring showed no measurable difference in the dormancy of the two varieties.

### CRITICAL PERIOD

The alfalfa plants frozen in the soil which were brought in during January for the two years, gave no marked evidence of killing during the winter. Roots dug for freezing point determination and for sap expression were uninjured throughout the two winters. Dead plants first appeared at the time of the spring thaw in 1923 and 1924. One sample for chemical determinations was taken February 7, 1925. At this time no dead plants were observed. During the thawing period,

March 18 to 27, thermo-electric readings were taken daily. On March 21 the first dead roots were observed and the number increased daily until March 27. At this time there were more dead roots in the Kansas common than there were living ones. An occasional dead root appeared in the Grimm variety at the latter date. One might expect to find a similar critical period during the fall if unfavorable changes of temperature should occur suddenly. However, during the interval under observation no such condition obtained. For comparison of the effect of this winter on other crops, it may be mentioned that observations (3) made in January, 1923, with several varieties of red clover, showed that the tender southern European sorts were entirely killed at that date, while the more hardy northern European sorts were uninjured.


Plate I. Potted Plants of Grimm and Kansas Alfalfa Exposed at the Same Temperature During the Winter

Note the killed and weakened plants of the tender variety while the hardy variety shows practically no injury.

## KILLING POINT OF ALALFA PLANTS

During the first year the laboratory equipment for obtaining low temperatures consisted of a Dewar beaker containing petroleum ether into which a copper coil was fitted. The material to be frozen was placed in a tightly stoppered bottle and submerged in the ether bath. The temperature was regulated by admitting liquid carbon dioxide into the coil. By this method temperatures of —40.0° C. were obtainable and could be held practically constant. By the use of this device it was found that fresh roots brought in from the field showed distinct differences in the killing point of the two varieties (Plate I). On April 6, 1923, the Grimm plants frozen at 10.0° C. were either killed

or injured while the Kansas plants were all killed. At —5.0° C. the Grimm plants were uninjured while the Kansas plants were either killed or injured. During the winter of 1924-25 a large refrigerator was available in which electrically controlled temperatures lower than —30.0° C. were obtainable. Seedling plants sown in late July were grown in 6-inch flower pots for use in determining the killing point while in the soil mass. The results of freezing potted plants are given in Table I.

TABLE I

EFFECT OF LOW TEMPERATURES UPON ALFALFA PLANTS COLLECTED AT DIFFERENT TIMES AND DIFFERENCE IN KILLING POINT BETWEEN THE TWO VARIETIES

| Variety | Date | Minimum temperature | Time* | Results |
|---|---|---|---|---|
| | | °C. | Hours | |
| Grimm | 10/ 8/24 | —11.9 | 12 | Killed |
| Kansas | 10/ 8/24 | —11.9 | 12 | Killed |
| Grimm | 10/ 9/24 | — 4.5 | 12 | Uninjured |
| Kansas | 10/ 9/24 | — 4.5 | 12 | Uninjured |
| Grimm | 10/10/24 | —13.5 | 12 | Killed |
| Kansas | 10/10/24 | —13.5 | 12 | Killed |
| Grimm | 10/24/24 | — 9.6 | 12 | Injured |
| Kansas | 10/24/24 | — 9.6 | 12 | Killed |
| Grimm | 10/25/24 | —12.5 | 12 | Injured |
| Kansas | 10/25/24 | —12.5 | 12 | Killed |
| Grimm | 12/26/24 | —11.0 | 12 | Injured |
| Kansas | 12/26/24 | —11.0 | 12 | Badly injured and killed |
| Grimm | 2/ 3/25 | Checks | .. | Uninjured |
| Kansas | 2/ 3/25 | Checks | .. | Uninjured |
| Grimm | 2/ 3/25 | —22.0 | 12 | Slight injury |
| Kansas | 2/ 3/25 | —22.0 | 12 | Killed to badly injured |
| Grimm | 2/ 5/25 | —17.0 | 12 | Uninjured |
| Kansas | 2/ 5/25 | —17.0 | 12 | Killed |
| Grimm | 2/ 6/25 | — 8.0 | 12 | Uninjured |
| Kansas | 2/ 6/25 | — 8.0 | 12 | Uninjured |
| Grimm | 2/10/25 | —28.5 | 12 | Killed |
| Kansas | 2/10/25 | —28.5 | 12 | Killed |
| Grimm | 3/13/25 | — 8.0 | 12 | Uninjured |
| Kansas | 3/13/25 | — 8.0 | 12 | Injured |
| Grimm | 3/13/25 | Checks | .. | Uninjured |
| Kansas | 3/13/25 | Checks | .. | Uninjured |
| Grimm | 3/16/25 | —14.0 | 12 | Injured |
| Kansas | 3/16/25 | —14.0 | 12 | Badly injured to killed |
| Grimm | 3/18/25 | — 5.0 | 12 | Uninjured to slightly injured |
| Kansas | 3/18/25 | — 5.0 | 12 | Badly injured to killed |
| Grimm | 7/20/25 | — 6.0 | 12 | Killed |
| Kansas | 7/20/25 | — 6.0 | 12 | Killed |
| Grimm | 7/21/25 | — 3.0 | 12 | Killed |
| Kansas | 7/21/25 | — 3.0 | 12 | Killed |

* Trials showed that injury had reached its maximum and was not increased up to 24 hours.

It will be observed that there is a seasonal progression of cold resistance during the fall and early winter and a regression during the spring. For example, on October 8 both varieties were killed at —11.9° C., while on October 25 Grimm plants survived —12.5° C. but Kansas plants did not. It seems that the maximum cold resistance

for both varieties during February, 1925, was near —22.0° C. This resistance was lost during the early part of March, which was the time of the spring thaw. It is of interest to note that in the summer condition both varieties killed at —3.0° C. By comparing the data further, it will be seen that the Grimm variety endures lower temperatures than the Kansas variety under hardened conditions.

The data in Table II, supplied by the section of farm crops, were taken from thermograph records for the winter 1923-24.

TABLE II

TEMPERATURES AT SURFACE OF SOIL AND AT ONE INCH BELOW SURFACE

| Date | Air temperature at surface, minimum | Soil temperature 1 inch below surface, minimum | Surface covering |
|---|---|---|---|
| | °C. | °C. | |
| 12/21/23 | —7.2 | —2.2 | Snow |
| 1/23/24 | —3.9 | —3.3 | " |
| 2/ 4/24 | —1.1 | —1.7 | " |
| 3/13/24 | —4.4 | —2.8 | " |
| 3/24/24 | 0.0 | —2.2 | " |
| 4/ 2/24 | 0.0 | —1.7 | " |
| 4/21/24 | +3.3 | +1.7 | No snow |

From the data presented it is obvious that snow protects the plants from low temperatures as well as from sudden fluctuations. Goeppert (17) recorded similar observation in 1830. He also observed that roots of hardy plants killed at —12.5 to —18.7° C.

From the winter-killing point of alfalfa plants, as indicated in Table I, it is apparent that a covering of snow provides certain protection to these plants against winter killing. As previously shown, the resistance to low temperature is appreciably decreased during March thaws. In addition, it has been found by thermograph records at University Farm that when the surface is bare the daily minimum soil temperature near the surface is approximately as low as that of the atmosphere.

Roots killed by low temperature are readily detected, as they appear watery when cut in cross-section. When the sections are squeezed between the fingers, sap readily exudes at the cut surface, while living roots show no exudation under similar treatment. Dead roots readily disintegrate when thawed out, so that soon after the spring thaw they become moldy and decay. Evidence of the relative frost resistance of the crown tissue and root tissue is of interest at this point. Under field conditions as well as under controlled greenhouse conditions, it was frequently observed that the crown buds began to unfold but soon died back. This was caused by dead root tissue. Examination of weakened plants revealed that the central region of the root was browned and later decayed, leaving a hollow root (Plate II). Chandler

(11) found that the pith cells of young twigs of apple killed first as the result of severe cold. Root injury was observed under field and controlled greenhouse conditions. Goeppert, and more recently Apelt and others, observed that repeated freezing finally killed plant tissue. This, however, may be due to disturbed metabolism under experimental conditions rather than to low temperatures. It may well be questioned whether repeated freezing and thawing occurs under natural conditions in the soil.

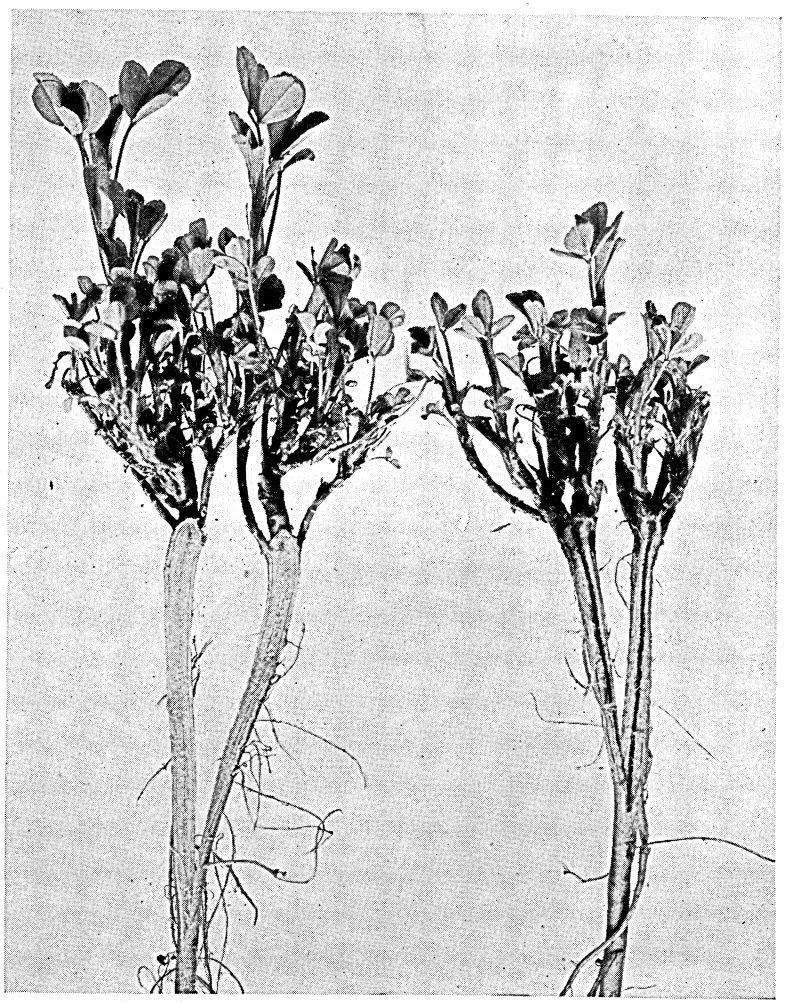


Plate II. Effect of Winter Killing on Kansas Common Alfalfa
(Photo May 5, 1923)
Left, Injured; Right, Uninjured.

## FREEZING POINT DEPRESSION OF ROOT TISSUE—THERMO-ELECTRIC METHOD

In freezing all samples, the bath was held as near —10.0° C. as possible. Sections of alfalfa root approximately one-fourth inch long were used. A hole, made into the tissue with a pin, permitted the firm insertion of the thermo-couple. All samples were allowed to undercool —1.5 to —2.0° C. Within this temperature range, inoculation was induced by jarring the apparatus. The advantage of considerable undercooling lies in the resulting rebound which facilitates obtaining a freezing point that is maintained for about a minute. The Leeds and Northrup Type K potentiometer was used for determining the potentials. A summary of the data is presented in Table III and Chart I, Figure A. A study of the results shows that in general no absolute correlation exists between the degree of the freezing point depression of the tissue and resistance to killing by freezing. For example, on December 29, 1922, the freezing point depression of the tissue for the Kansas variety was —3.2° C. while on July 5, 1923, the depression was —3.3° C. for the same variety. It is known from data previously presented that the actual killing points would be approximately —15.0° C. for the winter sample while it is known to be at —3.0° C. and undoubtedly higher, for the summer sample. This is in accord with the data presented by Rein (37), who found that no relation existed between cell turgor and its killing point. Harvey and Regeimbal (25), working with several species of trees and shrubs, found that in July the killing point was practically coincident with the freezing point. During the autumn the killing point gradually dropped until in November it had reached —27.0° C. while the freezing point remained fairly constant.

It will also be observed that the freezing point depression of the tissue is not so great during the winter months as in late autumn and early spring. In general, the seasonal trend of the freezing point depression of the tissue for the two varieties is similar, altho certain fluctuations occur. Close examination shows that for the critical period each spring Grimm had a somewhat greater depression. This difference was lost after the ground had thawed and growth had begun. While this difference is not great, it was found to prevail during each of the three years in which the study was carried out.

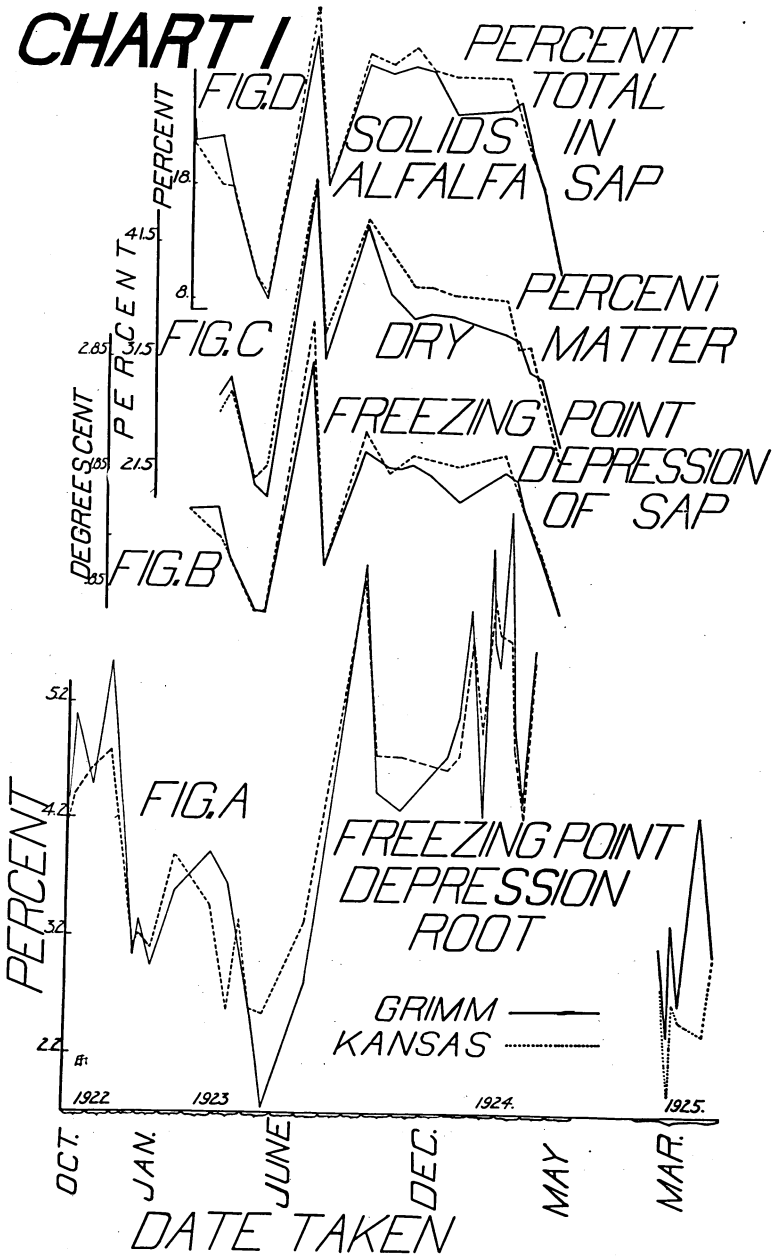
TABLE III

INFLUENCE OF ENVIRONMENT UPON FREEZING POINT DEPRESSION OF ROOT TISSUE OF ALFALFA AS DETERMINED BY THE THERMO-ELECTRIC METHOD

| Date | Kansas | | Grimm | |
|---|---|---|---|---|
| | Δ | Osmotic pressure atmospheres | Δ | Osmotic pressure atmospheres |
| 10/10/22 | 4.172 | 49.93 | 4.469 | 53.49 |
| 10/16/22 | 4.422 | 52.89 | 5.099 | 60.96 |
| 11/ 3/22 | 4.643 | 55.51 | 4.477 | 53.61 |
| 11/26/22 | 4.783 | 57.17 | 5.553 | 66.29 |
| 12/22/22 | 3.099 | 37.30 | 3.036 | 36.47 |
| 12/29/22 | 3.212 | 38.50 | 3.340 | 40.05 |
| 1/16/23 | 3.098 | 37.30 | 2.943 | 35.27 |
| 2/17/23 | 3.899 | 46.71 | 3.571 | 42.79 |
| 3/26/23 | 3.461 | 41.48 | 3.920 | 46.95 |
| 4/14/23 | 2.566 | 30.86 | 3.658 | 43.88 |
| 4/23/23 | 3.336 | 39.93 | 3.093 | 37.06 |
| 5/ 5/23 | 2.573 | 30.86 | 2.522 | 30.26 |
| 5/19/23 | 2.534 | 30.38 | 1.741 | 20.92 |
| 7/ 5/23 | 3.321 | 39.81 | 2.793 | 33.48 |
| 10/ 1/23 | 6.266 | 74.73 | 6.358 | 75.81 |
| 10/18/23 | 4.753 | 56.81 | 4.437 | 53.13 |
| 11/ 8/23 | 4.741 | 56.69 | 4.275 | 51.23 |
| 1/ 9/24 | 4.639 | 55.51 | 4.737 | 56.69 |
| 1/26/24 | 4.754 | 56.81 | 5.082 | 60.72 |
| 2/ 2/24 | 5.592 | 66.76 | 5.978 | 71.37 |
| 2/ 4/24 | 5.758 | 68.77 | 5.936 | 70.90 |
| 2/16/24 | 4.940 | 59.06 | 4.226 | 50.64 |
| 2/25/24 | 5.658 | 67.47 | 6.542 | 78.09 |
| 2/28/24 | 6.143 | 73.29 | 5.736 | 68.53 |
| 3/ 8/24 | 5.798 | 69.12 | 5.518 | 65.93 |
| 3/22/24 | 5.748 | 68.65 | 6.869 | 81.63 |
| 3/25/24 | 4.822 | 57.64 | 4.998 | 59.78 |
| 4/ 1/24 | 4.212 | 50.40 | 4.345 | 52.06 |
| 4/17/24 | 5.553 | 66.29 | 5.651 | 67.47 |
| 3/18/25 | 2.754 | 33.00 | 3.099 | 37.18 |
| 3/19/25 | 1.859 | 22.24 | 2.351 | 28.23 |
| 3/20/25 | 2.628 | 31.57 | 3.311 | 39.69 |
| 3/21/25 | 2.473 | 29.66 | 2.613 | 31.33 |
| 3/25/25 | 2.354 | 28.23 | 4.229 | 50.64 |
| 3/27/25 | 3.020 | 36.23 | 3.017 | 36.23 |

## FREEZING POINT DEPRESSION OF SAP

The readings for freezing point depression were made on expressed and centrifuged sap. Previous investigators (15, 20) have shown that the freezing point depression of press juice prepared from unfrozen tissue was less than that of press juice prepared from tissue which had been frozen previously. As the maximum freezing point depression of a sap was obtained from prefrozen material, and as relative checks were thus obtainable, it was assumed that such sap represented the cell contents. Harvey (23) presents data on the freezing point depression of cabbage juice obtained by prefreezing the tissue and by expressing without prefreezing. He concludes that juices expressed from frozen tissues do not represent the true concentration of the cell sap for all of its constituents. Newton, Brown, and Martin (35)




Physical Data of Root Tissue and Expressed Sap

report the results of a study concerning the methods of extracting a plant press juice and its utility in physiological studies. They say: "Undoubtedly pre-freezing of the tissues is desirable when only the osmotic pressure and conductivity of the fluids are to be determined, though clearly it does not obviate the necessity of standardizing the procedure followed in pressing out. When, however, the object is to obtain the cell contents in a condition as nearly as possible unchanged, pre-freezing is impracticable. Specifically, its precipitating effect on proteins necessitates its omission when the juice is to be used for the study of colloidal properties or protein distribution." Data are presented in their paper which show that previous treatment of the tissue markedly affects the freezing point depression of the sap. After discussing the factors involved which may influence the freezing point depression of the sap, the writers make the following statement: "The experimental results given in Table III can not therefore be held to either prove or disprove the assumption that the press-juice has the same composition as the original tissue fluids." Data presented by Harvey and also by Newton seem to point definitely to the conclusion that a press juice obtained by prefreezing the tissues does not represent the true concentration of the cell sap for all of its constituents.

As a precision method, the Heidenhain cryoscope used on plant saps was more accurate in determining the freezing point of a given sample of sap than was the thermo-electric method in determining the freezing point of a section of a root of alfalfa. However, the question still to be answered is: What is a press juice in terms of the true cell contents? A Heidenhain cryoscope with a temperature range of $-7.5°$ C. was found to facilitate freezing point determinations. These data, shown below, do not correlate with the relative winter hardiness of the varieties nor with the winter hardened condition of the plants. Evidently the freezing point depression can not be used as a measure of winter hardiness. An examination of Table IV and Chart 1, Figures B and D, shows that the freezing point depression of the sap is closely correlated with the total solids, as shown by the refractometer readings. The greatest depression was obtained on July 26, when the plants were wilted because of drouth and the moisture content of the root tissue was low. The smallest depressions were obtained during the active growing period, when the moisture content was high and the total solids in the sap were low. This condition prevailed during May for the two years under study. It should be borne in mind that all root material used in this experiment was undoubtedly killed, as the temperature of solid carbon dioxide is $-79.0°$ C. under the conditions used, while the lowest killing point

of alfalfa roots investigated in the hardened condition was approximately —28.0° C. Newton (34) found a hardy variety of wheat the leaves of which apparently endured —40.0° C. in a hardened condition while the leaves of tender varieties were killed. Undoubtedly the sap obtained both from living and from killed leaf tissue accounted for some of the differences which he obtained.

TABLE IV

INFLUENCE OF ENVIRONMENT UPON FREEZING POINT DEPRESSION AND TOTAL SOLIDS IN PREPARED PRESS JUICE

| Date | Kansas | | | Grimm | | |
|---|---|---|---|---|---|---|
| | Freezing point depression | Total solids at 20° C. | Osmotic pressure atmospheres | Freezing point depression | Total solids at 20° C. | Osmotic pressure atmospheres |
| | °C. | Per cent | | °C. | Per cent | |
| 10/18/22 | 2.160* | .... | 25.95 | 2.148* | .... | 25.83 |
| 3/ 3/23 | 1.526 | 21.41 | 18.40 | 1.523 | 21.75 | 18.28 |
| 4/ 6/23 | 1.292 | 17.80 | 15.52 | 1.542 | 22.20 | 18.52 |
| 4/20/23 | 1.119 | 17.75 | 13.48 | 1.102 | 17.25 | 13.24 |
| 5/ 5/23 | 0.636 | 10.00 | 7.71 | 0.648 | 9.96 | 7.83 |
| 5/19/23 | 0.649 | 8.50 | 7.83 | 0.634 | 8.00 | 7.59 |
| 7/26/23 | 3.156 | 33.50 | 37.91 | 2.784 | 30.80 | 33.36 |
| 8/ 7/23 | 1.041 | 17.90 | 12.52 | 1.038 | 18.10 | 12.52 |
| 10/ 2/23 | 2.192 | 29.25 | 26.31 | 2.023 | 28.43 | 24.28 |
| 10/27/23 | 1.831 | 29.90 | 22.00 | 1.887 | 28.30 | 22.72 |
| 11/12/23 | 1.900 | 28.50 | 22.84 | 1.876 | 27.70 | 22.60 |
| 11/27/23 | 1.986 | 22.80 | 23.92 | 1.905 | 25.20 | 22.96 |
| 12/21/23 | 1.960 | 28.00 | 23.56 | 1.823 | 27.80 | 21.88 |
| 1/19/24 | 1.908 | 27.50 | 22.96 | 1.599 | 24.20 | 19.24 |
| 3/13/24 | 1.978 | 27.40 | 23.80 | 1.856 | 24.60 | 22.36 |
| 3/24/24 | 1.701 | 24.10 | 20.44 | 1.789 | 25.30 | 21.52 |
| 4/ 2/24 | 1.525 | 20.55 | 18.40 | 1.547 | 20.82 | 18.64 |
| 4/21/24 | 1.138 | 17.90 | 13.72 | 1.116 | 17.70 | 13.48 |
| 5/17/24 | 0.653 | 10.42 | 7.83 | 0.645 | 10.57 | 7.83 |
| 2/ 7/25 | 1.457 | 20.04 | 17.56 | 1.432 | 19.66 | 17.20 |

* Leaf sap.

## TOTAL SOLIDS IN SAP

These determinations were made under uniform conditions at 20° C. and are recorded in Table IV and Chart 1, Figure D. No relation exists between the winterhardiness of these two varieties and the percentage of total solids. The highest reading of total solids was obtained July 26, 1923, when the soil was dry enough to cause the plants to wilt. There appears to be a positive correlation between the available moisture in soils and the percentage of total solids in the sap. This is in line with the general theory of hydrophyllic colloids.

## VISCOSITY OF SAP

These determinations were made with a viscosimeter of the Ostwald type, in which the sap flowed through a capillary at a constant temperature of 20.0°C. The data in Table V represent the time in seconds required for 1.5 cc. of sap to flow through the capillary. By referring to the data on total solids for the same samples, it will be

seen that a positive correlation exists between the percentage of total solids and viscosity, but no consistent differences exist for the two varieties under study.

TABLE V

INFLUENCE OF ENVIRONMENT UPON PERCENTAGE OF DRY WEIGHT IN ROOTS, PERCENTAGE OF TOTAL SOLIDS IN EXPRESSED JUICE, AND VISCOSITY OF EXPRESSED JUICE AT 20° C.

| Variety | Date | Dry weight in sample | Total solids in sap | Viscoscity (water = 126 sec.) |
|---|---|---|---|---|
| | | Per cent | Per cent | Seconds |
| Grimm | 3/ 1/23 | .... | 23.65 | .... |
| Kansas | 3/ 1/23 | .... | 22.80 | .... |
| Grimm | 3/ 3/23 | .... | 21.75 | .... |
| Kansas | 3/ 3/23 | .... | 21.41 | .... |
| Grimm | 4/ 6/23 | 28.13 | 22.20 | .... |
| Kansas | 4/ 6/23 | 26.62 | 17.80 | .... |
| Grimm | 4/20/23 | 29.72 | 17.25 | 601 |
| Kansas | 4/20/23 | 28.50 | 17.75 | 523 |
| Grimm | 5/ 5/23 | 20.31 | 9.96 | 255 |
| Kansas | 5/ 5/23 | 20.80 | 10.00 | 220 |
| Grimm | 5/19/23 | 19.26 | 8.00 | 279 |
| Kansas | 5/19/23 | 21.79 | 8.50 | 301 |
| Grimm | 7/26/23 | 45.99 | 30.80 | 1114 |
| Kansas | 7/26/23 | 46.70 | 33.50 | 1305 |
| Grimm | 8/ 7/23 | 31.34 | 18.10 | 748 |
| Kansas | 8/ 7/23 | 33.55 | 17.90 | 464 |
| Grimm | 10/ 2/23 | 42.95 | 28.43 | 775 |
| Kansas | 10/ 2/23 | 43.63 | 29.25 | 792 |
| Grimm | 11/12/23 | 36.99 | 27.70 | .... |
| Kansas | 11/12/23 | 40.77 | 28.50 | .... |
| Grimm | 11/27/23 | 34.77 | 28.30 | .... |
| Kansas | 11/27/23 | 37.62 | 29.90 | .... |
| Grimm | 12/21/23 | 35.25 | 27.80 | 676 |
| Kansas | 12/21/23 | 37.63 | 28.00 | 823 |
| Grimm | 1/19/24 | 35.04 | 24.20 | .... |
| Kansas | 1/19/24 | 36.93 | 27.50 | .... |
| Grimm | 3/13/24 | 33.55 | 24.60 | .... |
| Kansas | 3/13/24 | 36.50 | 27.40 | .... |
| Grimm | 3/24/24 | 33.17 | 25.30 | .... |
| Kansas | 3/24/24 | 32.34 | 24.10 | .... |
| Grimm | 4/ 4/24 | 30.32 | 20.82 | .... |
| Kansas | 4/ 4/24 | 32.59 | 20.55 | .... |
| Grimm | 4/21/24 | 29.72 | 17.70 | .... |
| Kansas | 4/21/24 | 28.50 | 17.90 | .... |
| Grimm | 5/17/24 | 23.82 | 10.57 | .... |
| Kansas | 5/17/24 | 22.67 | 10.42 | .... |
| Grimm | 2/ 7/25 | 32.26 | 19.66 | .... |
| Kansas | 2/ 7/25 | 32.97 | 20.04 | .... |

## BOUND WATER AS A MEASURE OF WINTER RESISTANCE

The press juice was obtained from roots which were prepared according to the method previously described. The centrifuged sap was kept on ice and determinations were completed within eight hours. Newton and Gortner (33) present a method of estimating the hydrophyllic colloid content of expressed plant tissue fluids. In brief, the

procedure is as follows: The freezing point depression of a freshly prepared plant press juice is determined. Then the total solids are determined by the refractometer, using the method proposed by Gortner and Hoffman (21). Using the total solids as a basis, just enough press juice is weighed out to contain 10 grams of water, to which exactly 3.422 grams of pulverized sucrose is added. After this is dissolved, the freezing point depression is determined and is usually found to have increased more than the theoretical 2.085° C., allowing for the formation of sucrose hexahydrate. It is assumed that the excess is a measure of the bound water held by cell colloids and therefore not available for the solution of sugar. The application of this method to a limited number of samples showed no correlation between the bound water of the varieties in a hardened condition and winter hardiness, nor did it give a strikingly higher percentage of bound water for the samples taken during winter and early spring, as shown in Table VI. The symbols used as headings for columns 4, 5, 6, and 7 are the same as those used by Newton and Gortner (33).

TABLE VI

INFLUENCE OF ENVIRONMENT UPON PERCENTAGE OF TOTAL SOLIDS, FREEZING POINT DEPRESSION, AND BOUND WATER IN PLANT PRESS JUICE OF TWO VARIETIES OF ALFALFA

| Variety | Date | Total solids | $\Delta$* | $\Delta a$ | $\Delta a - \Delta$ | $\Delta a - (\Delta + Km)$ | Bound water |
|---|---|---|---|---|---|---|---|
| | | Per cent | | | | | Per cent |
| Grimm | 11/12/23 | 27.7 | 1.876 | 4.370 | 2.494 | 0.409 | 14.6 |
| Kansas | 11/12/23 | 28.5 | 1.900 | 4.383 | 2.483 | 0.398 | 14.3 |
| Grimm | 11/27/23 | 25.2 | 1.913 | 4.289 | 2.376 | 0.291 | 10.9 |
| Kansas | 11/27/23 | 22.8 | 1.982 | 4.509 | 2.527 | 0.442 | 15.6 |
| Grimm | 1/19/24 | 24.2 | 1.554 | 3.901 | 2.347 | 0.262 | 9.9 |
| Kansas | 1/19/24 | 27.5 | 1.911 | 4.366 | 2.455 | 0.370 | 13.3 |
| Grimm | 3/13/24 | 24.6 | 1.859 | 4.310 | 2.451 | 0.366 | 13.3 |
| Kansas | 3/13/24 | 27.4 | 1.987 | 4.544 | 2.566 | 0.481 | 16.5 |
| Grimm | 3/24/24 | 25.3 | 1.702 | 4.120 | 2.418 | 0.333 | 12.4 |
| Kansas | 3/24/24 | 24.1 | 1.792 | 4.183 | 2.391 | 0.306 | 11.4 |
| Grimm | 4/ 4/24 | 20.8 | 1.540 | 3.931 | 2.391 | 0.306 | 11.4 |
| Kansas | 4/ 4/24 | 20.6 | 1.492 | 3.983 | 2.491 | 0.406 | 14.5 |
| Grimm | 4/21/24 | 17.7 | 1.077 | 3.364 | 2.287 | 0.202 | 7.9 |
| Kansas | 4/21/24 | 17.9 | 1.083 | 3.371 | 2.287 | 0.202 | 7.9 |
| Grimm | 5/17/24 | 10.6 | 0.641 | 2.941 | 2.300 | 0.215 | 8.4 |
| Kansas | 5/17/24 | 10.4 | 0.658 | 2.982 | 2.324 | 0.239 | 9.2 |

*$\Delta$ Freezing point depression of freshly expressed juice.

$\Delta^a$ Freezing point depression after the addition of the sugar.

$\Delta a - \Delta$ Actual added depression due to added sugar.

$\Delta a(\Delta + Km)$ This is equal to $(\Delta a - \Delta) - 2.085$, the amount by which the depression found on the addition of sugar is in excess of that expected on theoretical grounds.

The percentage of bound water is calculated by the formula, Bound water $= \frac{\Delta a\,(\Delta + Km)\,89.2}{\Delta a - \Delta}$, where 89.2 is the percentage of free water in a free molar solution of sucrose. Scatchard (39) has studied the hydration of sucrose in the light of previous investigations. He found strong evidence of the presence of a hexahydrate. While

vapor pressure measurements apparently did not agree with the presence of either a hexahydrate or a heptahydrate alone, he concluded that in view of all possible errors a hexahydrate met the conditions best.

Newton found a close correlation between bound water and winter hardiness of wheat varieties. He further measured the hydrophyllic colloid content of Cereus and Bryophyllum which were grown in the greenhouse. The results indicate a high hydrophyllic colloid content for Cereus and a total absence of such colloids in Bryophyllum. He also found that gum arabic, when added to water, acts as a hydrophyllic colloid in direct proportion to the amount added. Data presented in Table VI give evidence of the presence of hydrophyllic colloids in alfalfa roots. As previously stated, this measurement does not differentiate the hardy from the non-hardy variety.

## VOLUME OF PRESS JUICE

The volume of press juice was taken according to the procedure of Newton. The data presented in Table VII show no relation to known hardiness of the material. As a measure of difference in imbibitional force which has been used by Newton in wheat, this method has no significance in relation to the material at hand.

TABLE VII

VOLUME OF PLANT PRESS JUICE PER 100 GRAMS OF FROZEN TISSUE OBTAINED AT VARIOUS PRESSURES IN RELATION TO THE TIME OF SAMPLING

| Variety | Date | Kilograms per square centimeter pressure | | | | |
|---|---|---|---|---|---|---|
| | | 50 | 100 | 200 | 300 | 400 |
| | | cc. | cc. | cc. | cc. | cc. |
| Grimm | 11/12/23 | 8.2 | 9.0 | 9.4 | 11.2 | 11.4 |
| Kansas | 11/12/23 | 3.9 | 4.2 | 4.5 | 4.7 | 4.9 |
| Grimm | 11/26/23 | 16.2 | 18.8 | 21.0 | 21.0 | 21.0 |
| Kansas | 11/26/23 | 17.3 | 22.2 | 22.4 | 22.4 | 22.4 |
| Grimm | 11/27/23 | 11.7 | 15.2 | 16.1 | 16.2 | 16.2 |
| Kansas | 11/27/23 | 6.1 | 7.3 | 8.0 | 8.0 | 8.0 |
| Grimm | 12/21/23 | 7.5 | 8.9 | 10.4 | 11.0 | 11.5 |
| Kansas | 12/21/23 | 6.4 | 7.5 | 8.2 | 8.4 | 8.4 |
| Grimm | 1/19/24 | 13.5 | 21.6 | 26.3 | 27.5 | 28.3 |
| Kansas | 1/19/24 | 10.4 | 12.0 | 13.0 | 14.1 | 14.3 |
| Grimm | 3/13/24 | 15.2 | 19.7 | 25.8 | 27.7 | 28.0 |
| Kansas | 3/13/24 | 8.7 | 12.5 | 14.5 | 15.6 | 16.1 |
| Grimm | 3/24/24 | 10.8 | 14.0 | 17.0 | 18.2 | 18.9 |
| Kansas | 3/24/24 | 11.5 | 14.8 | 16.2 | 17.3 | 17.6 |
| Grimm | 4/ 2/24 | 20.0 | 22.5 | 25.0 | 26.0 | 26.5 |
| Kansas | 4/ 2/24 | 20.5 | 24.3 | 32.4 | 34.5 | 35.6 |
| Grimm | 4/21/24 | 14.8 | 18.4 | 20.5 | 21.5 | 22.1 |
| Kansas | 4/21/24 | 14.8 | 17.0 | 19.3 | 20.0 | 20.0 |
| Grimm | 5/17/24 | 17.8 | 20.9 | 24.0 | 25.1 | 26.3 |
| Kansas | 5/17/24 | 33.0 | 42.0 | 45.3 | 47.3 | 49.3 |
| Grimm | 2/ 7/25 | 21.0 | 27.4 | 31.5 | 33.2 | 34.0 |
| Kansas | 2/ 7/25 | 26.7 | 31.3 | 35.4 | 36.3 | 37.3 |

## CHEMICAL DETERMINATIONS

### PREPARATION OF SAMPLES FOR ANALYSIS

Dry weight determinations were made on duplicate samples of approximately 10 grams of fresh material which were dried to constant weight in an electrically heated oven at 100.0° C. The total organic nitrogen determinations were made by the Kjeldahl-Gunning method, using the residues from the dry weight samples.

*Extraction of sugars and soluble nitrogen.*—The preserved alcoholic samples were extracted on a steam bath in large Landsiedl extractors until the Molish test became negative. The volume and concentration of alcohol were approximately maintained by the occasional addition of small quantities of 95 per cent alcohol. Extraction was usually complete within from 18 to 24 hours. The extract was removed from the bath and the diluted alcoholic portion was made up to 500 cc. volume in water. After transferring to a large separatory funnel, 20 cc. of chloroform was added. This was thoroly shaken and set aside overnight. The chloroform containing the lipoid material was then drawn off. Following this a 10-cc. portion of chloroform was added and later drawn off. The chloroform extract containing the lipoid material was dried on a steam bath and weighed. No quantitative significance is given to this fraction.

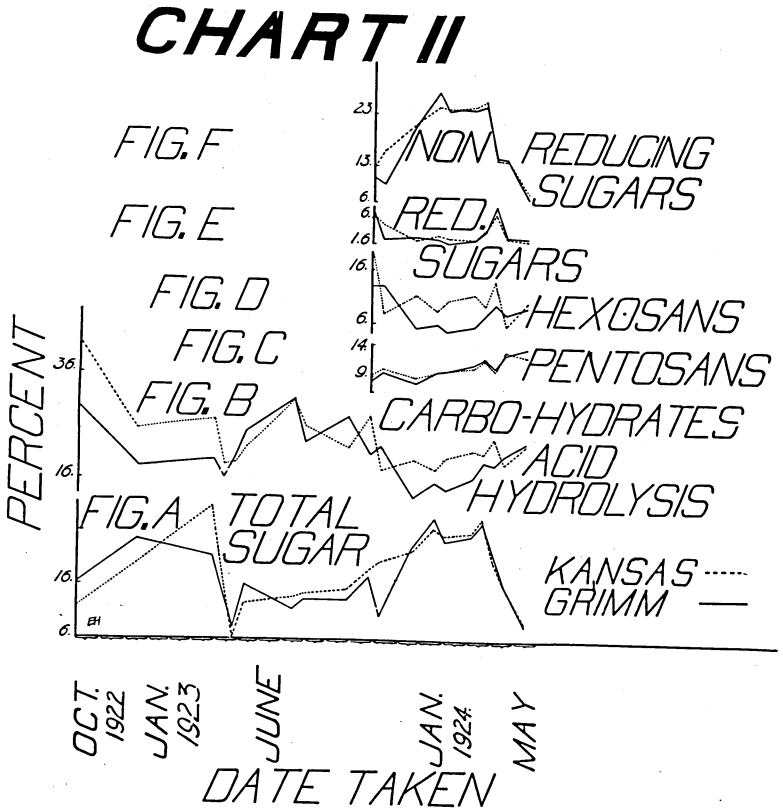
The extract was concentrated in the Claissen (45) apparatus under a reduced pressure of 20 to 25 mm. with the distillation flask in a water bath at a temperature from 40.0° to 60.0° C. Each sample was previously divided into two equal 250-cc. aliquots. The first aliquot, used for nitrogen determinations, was evaporated to a thick viscous mass in order to assure the removal of all alcohol. Later it was transferred and made up to a 100-cc. volume in hydrochloric acid (1:5) for nitrogen determinations. The other aliquot, which was used for sugar determinations, was reduced to approximately 25 cc. It was then taken up in approximately 170 cc. volume with distilled water and transferred to a 250-cc. volumetric flask previous to clarification,

*Clearing extract for sugar analysis.*—According to the method proposed by Horne (27), powdered neutral lead acetate was added in small quantities until no additional precipitate was formed. The solution was then allowed to stand until coagulation was completed. After this di-sodium phosphate was added in small quantities to precipitate the excess lead. Before filtering, sufficient water was added to bring the volume up to 250 cc. The clear filtrate was then ready for sugar determination.

## DETERMINATIONS AND RESULTS

*Soluble carbohydrates.*—Soluble carbohydrates were extracted by alcohol and determined as total sugar and reducing sugar.

Total sugar.—Of the clarified filtrate, 75 cc. was placed in a 100-cc. volumetric flask to which 5 cc. of concentrated hydrochloric acid was added. This was then set away to wait for inversion at room temperature for 24 hours. Thereupon the contents were nearly neutralized with strong sodium hydroxide. It was found that this point could be readily observed by color change of the diluted extract. Neutralization was then completed with pure sodium carbonate. Following this, the volume was made up to 100 cc. and the reducing sugars were determined in triplicate 10-cc. portions. The copper determinations were carried out according to the thiosulphate titration method as given in the Official Methods of Analysis of the Association of Official Agricultural Chemists. The data in Table VIII and Chart II, Figure A, give the results based upon the material and method used in estimating total sugars.




Carbohydrate Data Calculated in Per Cent of Dry Weight

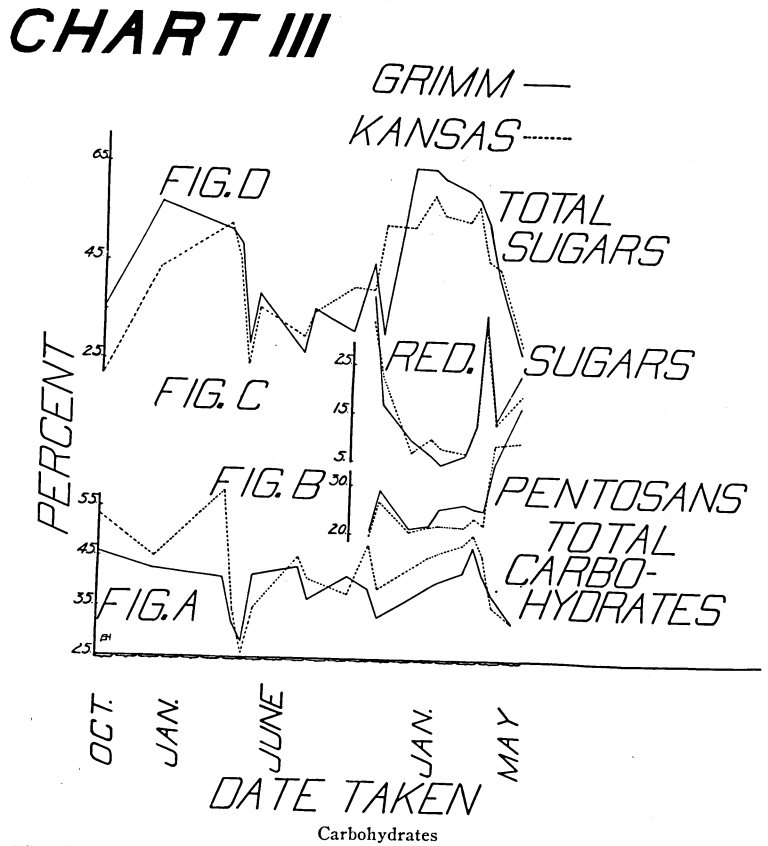
## TABLE VIII

EFFECT OF THE ENVIRONMENT UPON THE CARBOHYDRATES IN ALFALFA ROOTS CALCULATED ON THE DRY WEIGHT BASIS

| Date | Kansas | | | | | | Grimm | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Total carbohydrates in residue. Acid hydrol. | Total sugars | Reducing sugars | Non-reducing sugar | Pentosans | Hexosans | Total carbohydrates in residue. Acid hydrol. | Total sugars | Reducing sugars | Non-reducing sugar | Pentosans | Hexosans |
| | Per cent | Per cent | Per cent | Per cent | Per cent | Per cent | Per cent | Per cent | Per cent | Per cent | Per cent | Per cent |
| 10/19/22 | 41.45 | 11.91 | .... | .... | .... | .... | 29.49 | 16.47 | .... | .... | .... | .... |
| 12/26/22 | 25.20 | 19.81 | .... | .... | .... | .... | 18.27 | 24.34 | .... | .... | .... | .... |
| 4/ 6/23 | 27.40 | 30.97 | .... | .... | .... | .... | 19.63 | 21.35 | .... | .... | .... | .... |
| 4/23/23 | 18.87 | 15.81 | .... | .... | .... | .... | 16.37 | 15.70 | .... | .... | .... | .... |
| 5/ 5/23 | 19.25 | 6.31 | .... | .... | .... | .... | 20.23 | 8.11 | .... | .... | .... | .... |
| 5/19/23 | 22.30 | 12.69 | .... | .... | .... | .... | 25.24 | 16.18 | .... | .... | .... | .... |
| 7/26/23 | 31.42 | 13.75 | .... | .... | 7.98 | 23.44 | 31.41 | 11.67 | .... | .... | 8.88 | 22.53 |
| 8/ 7/23 | 26.31 | 14.47 | .... | .... | .... | .... | 23.45 | 13.26 | .... | .... | .... | .... |
| 10/ 2/23 | 22.51 | 15.33 | .... | .... | .... | .... | 28.15 | 13.23 | .... | .... | .... | .... |
| 10/30/23 | 28.47 | 19.19 | 6.59 | 12.60 | 9.36 | 19.11 | 21.27 | 17.76 | 6.98 | 10.78 | 8.19 | 13.08 |
| 11/10/23 | 18.06 | 20.74 | 4.71 | 16.03 | 10.38 | 7.68 | 22.87 | 10.36 | 1.80 | 8.56 | 9.71 | 13.16 |
| 12/21/23 | 20.14 | 22.88 | 1.61 | 24.49 | 8.91 | 11.23 | 13.06 | 23.59 | 2.31 | 21.28 | 7.86 | 5.20 |
| 1/23/24 | 18.29 | 27.10 | 2.71 | 24.39 | 9.89 | 8.40 | 15.79 | 29.03 | 1.95 | 27.08 | 9.88 | 5.91 |
| 2/ 4/24 | 20.54 | 25.89 | 2.12 | 23.77 | 10.26 | 10.28 | 14.59 | 24.64 | 1.16 | 23.48 | 10.03 | 4.56 |
| 3/13/24 | 21.96 | 26.36 | 1.86 | 24.50 | 10.55 | 11.41 | 16.68 | 25.74 | 1.80 | 23.94 | 11.23 | 5.45 |
| 3/24/24 | 21.39 | 29.10 | 3.45 | 25.65 | 12.02 | 9.37 | 19.73 | 28.04 | 3.45 | 24.59 | 12.30 | 7.43 |
| 4 /2/24 | 24.44 | 21.01 | 6.87 | 14.14 | 10.27 | 14.17 | 19.21 | 22.64 | 8.04 | 14.60 | 10.60 | 8.60 |
| 4/21/24 | 19.44 | 16.02 | 2.13 | 13.89 | 13.79 | 5.65 | 21.10 | 16.55 | 2.24 | 14.31 | 13.24 | 7.86 |
| 5/17/24 | 23.01 | 9.45 | 1.68 | 7.77 | 12.79 | 10.22 | 23.40 | 8.91 | 2.09 | 6.82 | 14.31 | 9.09 |
| 2/ 7/25 | 19.98 | 24.03 | 3.63 | 20.40 | 9.74 | 10.24 | 18.07 | 23.77 | 3.47 | 20.30 | 11.44 | 6.63 |

Reducing sugar.—Reducing sugars were determined directly on 25-cc. portions of the clarified and filtered extract. The same technic was used as is given under total sugars. The data are reported in Table VIII and in Chart II, Figure E.

*Insoluble carbohydrates in residue.*—This represents all carbohydrate material left in the residue after the extraction in hot alcohol. Two separations were made: (1) that fraction which is hydrolyzed by direct acid hydrolysis, as outlined in A. O. A. C. official methods, which includes besides starch the pentosans and other carbohydrates; (2) that fraction which is determined as pentosans according to official methods. For convenience these will be referred to as total insoluble carbohydrates and pentosans. The total insoluble carbohydrates were determined in duplicate on a 2-gram sample of the residue remaining after alcoholic extraction. The samples were brought to constant weight and hydrolyzed. The hydrolyzed samples were treated as previously indicated for total sugars. Pentosans were determined in duplicate on 1-gram samples and finally calculated from the phloro-




Carbohydrates

Fig. A, per cent of total carbohydrates in alfalfa roots. Figs. B, C, and D, expressed in percentages of total carbohydrates.

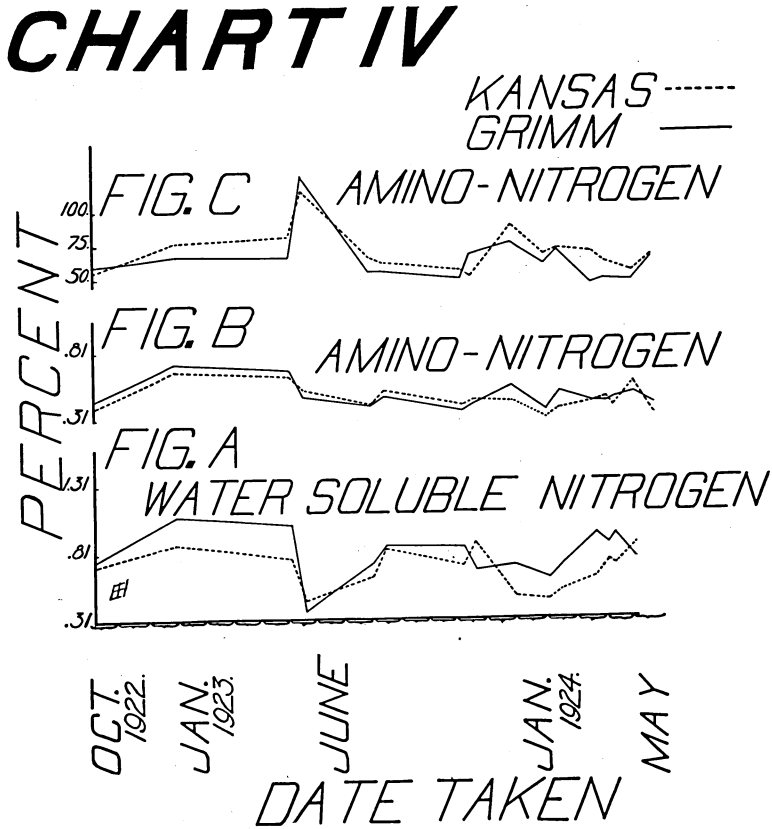
glucide precipitate, allowing for the proper solubility factor as given in A. O. A. C. official methods.

The results are found in Table VIII and Chart II, Figures B and C. Total carbohydrates other than pentosans have been determined by difference and indicated as hexosans. The greater part of this may be considered starch.

*Nitrogenous material.*—This is divided into three fractions, namely, the total organic nitrogen in the dry weight residue, the total alcohol-soluble organic nitrogen, and the amino-nitrogen extracted in alcohol.

The total organic nitrogen in the dry weight residues is available only in part, owing to the loss of the residues in storage.

The total alcohol-soluble organic nitrogen was determined on 25-cc. portions of the concentrated extract prepared as for amino nitrogen. The results are presented in Table X and Chart IV, Figure A. The difficulties encountered in determining nitrate nitrogen in solutions have been thoroly discussed by Bristol and Page (9). While it is assumed




Nitrogenous Material

Figs. A and B are percentages on dry weight basis. Fig. C is amino-nitrogen expressed in per cent of total water-soluble nitrogen.

that nitrate nitrogen may occur in alfalfa root material, no determinations have been made.

Amino nitrogen was determined by use of the Van Slyke apparatus (45), using 10-cc. portions of the filtered extract. Preliminary trials gave an appreciably higher yield when deaminization was continued one hour rather than the usual 6 minutes. Thus the data reported are based upon the amount of nitrogen liberated in one hour. The data are presented in Table X and Chart IV, Figure B.

## DISCUSSION

### CARBOHYDRATE RESERVES IN RELATION TO OVERWINTERING OF ALFALFA

From the data shown in Table VIII it is obvious that large quantities of carbohydrate reserves are stored in the roots of alfalfa plants. These reserves are drawn upon largely during the period of early spring growth. Nelson (31) has pointed out that low carbohydrate root reserves are one cause of winter killing in alfalfa. More specifically, he emphasizes the danger of cutting so late in autumn that there would not be sufficient leaf surface or time before freezing weather for the plant to accumulate ample reserves. However, he does not indicate quantitatively what relative amounts of reserves are necessary for overwintering. Wahlen (46) found that the root reserves were not entirely used in early spring, which is in accord with data presented in this report.

Fisher (16) was the first to make an extensive study of the carbohydrate reserves in woody tissues. He found that in late autumn the starch in the cortical region was converted into sugar and in early spring it was reconverted into starch. Lidforss (28) examined a wide range of species of wintergreen plants in southern Sweden, and found that the leaf tissue usually was entirely free from starch during winter. He also recognized that certain plants do not store starch but only sugar. He further found that submerged aquatic plants which never reached 0.0° C. contained starch throughout their tissues. Those which float on the surface of the water showed no starch in the upper epidermis and little in the mesophyll, while the lower epidermis and adjacent tissue were rich in starch. Fischer found that the iris rootstock was starch-free in the periphery, but internally starch was abundant during winter. From Lidforss' work the theory (advanced later by Schaffnit, 40, 41, 42) was evolved that sugar has a protective effect against the precipitation of plant proteins. An examination of Table VIII and Charts II and III reveals that in general the change from starch to sugar takes place in alfalfa roots, as shown by the higher sugar content, during the winter months. Zacharowa (51), in examining non-hardened seedling root tissues, presents data which he

uses to refute Lidforss' theory relative to the protective effect of sugar against protein precipitation. The phenomenon of sugar formation has been examined by Fischer and shown to be the result of temperature change. He reports 5.0° C. as a starch regeneration minimum. Coville (12) advanced the following theory in explanation of the formation of sugar during the process of chilling: "The starch grains stored in the cells of the plant are at first separated by the living and active cell membranes from the enzyme that would transform the starch into sugar, but when the plant is chilled the vital activity of the cell membrane is weakened so that the enzyme 'leaks' through it, comes in contact with the starch, and turns it into sugar." Apelt (2), in his investigation on the freezing of potato tubers, found that the increase in sugar content resulting from previous exposure to cold above freezing was too small to account for the increased freezing point depression. Apelt's results agree with those of Rein (37).

When the total sugars are calculated in terms of total carbohydrates (Table IX and Chart III, Figure D) it appears that the sugar ratio is practically doubled in winter. By comparing the percentages of the two varieties, it is seen that the Grimm variety contains a relatively higher percentage of sugar. This may be sufficient to be considered as one point of advantage on the assumption that sugar acts as a protective colloid. Since reducing sugars are practically equal in the two varieties under consideration, this can not be used as a point of differentiation. A well known exception to Lidforss' theory of the protective action of sugar is found in the sugar beet, which is not winter hardy.

Pentosan content has been correlated with hardiness by Hooker (26) and Rosa (38). This, however, has been refuted recently by McGinty (30). From the data here presented, no significant difference in pentosan content exists between varieties. While there is an increase in April and May, based on dry weight, it is also shown that other carbohydrate reserves are low. This appears to indicate a difference in their availability during this active growth period as compared with starch and sugar. Spoehr (43) has shown that in cacti low water content and high temperature are associated with pentosan content. He arrives at the conclusion that under stress the plant possesses the power of utilizing the reserve polysaccharides, including pentosans. Under unfavorable conditions the latter carbohydrates will be utilized. The data indicate that this occurs in alfalfa. As the more available hexoses and hexosans are utilized first, it seems logical that the less available pentosans should increase on a dry weight basis during early spring growth.

TABLE IX

CARBOHYDRATES IN ALFALFA ROOTS EXPRESSED IN PERCENTAGES OF TOTAL CARBOHYDRATES

| Date | Kansas | | | | Grimm | | | |
|---|---|---|---|---|---|---|---|---|
| | Total carbohydrates | Total sugar in terms of total carbohydrates | Reducing sugar based on total sugar | Pentosans based on total carbohydrates | Total carbohydrates | Total sugar in terms of total carbohydrates | Reducing sugar based on total sugar | Pentosans based on total carbohydrates |
| | Per cent | Per cent | Per cent | Per cent | Per cent | Per cent | Per cent | Per cent |
| 10/19/22 | 53.36 | 22.3 | ... | ... | 45.96 | 35.8 | ... | ... |
| 12/26/22 | 45.01 | 44.0 | ... | ... | 42.61 | 57.1 | ... | ... |
| 4/ 6/23 | 58.37 | 53.0 | ... | ... | 40.98 | 52.1 | ... | ... |
| 4/23/23 | 34.68 | 45.5 | ... | ... | 32.07 | 48.9 | ... | ... |
| 5/ 5/23 | 25.56 | 24.6 | ... | ... | 28.34 | 28.8 | ... | ... |
| 5/19/23 | 34.99 | 36.2 | ... | ... | 41.42 | 39.0 | ... | ... |
| 7/26/23 | 45.17 | 30.4 | ... | 17.6 | 43.08 | 27.1 | ... | 20.6 |
| 8/ 7/23 | 40.78 | 35.4 | ... | ... | 36.71 | 36.1 | ... | ... |
| 10/ 2/23 | 37.84 | 40.5 | ... | ... | 41.38 | 31.9 | ... | ... |
| 10/30/23 | 47.66 | 40.2 | 34.3 | 19.8 | 39.03 | 45.5 | 39.2 | 20.9 |
| 11/10/23 | 38.80 | 53.4 | 22.7 | 26.7 | 33.23 | 31.1 | 17.3 | 29.2 |
| 12/21/23 | 43.02 | 53.1 | 7.1 | 20.7 | 36.65 | 64.8 | 9.8 | 21.4 |
| 1/23/24 | 45.39 | 59.7 | 10.0 | 21.7 | 44.82 | 64.7 | 6.7 | 22.0 |
| 2/ 4/24 | 46.43 | 55.7 | 8.2 | 22.1 | 39.23 | 62.8 | 4.7 | 25.5 |
| 3/13/24 | 48.22 | 54.4 | 7.1 | 21.8 | 42.42 | 60.6 | 6.9 | 26.4 |
| 3/24/24 | 50.49 | 57.6 | 11.8 | 23.8 | 47.77 | 58.6 | 12.3 | 25.7 |
| 4/ 2/24 | 45.45 | 46.2 | 32.6 | 22.5 | 41.85 | 54.1 | 35.5 | 25.3 |
| 4/21/24 | 35.46 | 45.1 | 13.3 | 38.8 | 37.65 | 43.9 | 13.5 | 35.1 |
| 5/17/24 | 32.46 | 29.1 | 17.7 | 39.4 | 32.31 | 27.5 | 23.4 | 44.2 |
| 2/ 7/25 | 43.01 | 55.8 | 15.1 | 22.1 | 41.84 | 56.8 | 14.6 | 27.3 |

## NITROGENOUS MATERIALS IN RELATION TO OVERWINTERING IN ALFALFA

The total organic nitrogen in the dry weight residue, available for a limited number of samples only, is useful to compare the amino-nitrogen to the total organic nitrogen. In this way it is possible to arrive at an estimate of the relative amount of protein splitting which occurred during winter. The data are presented in Table X and Chart IV.

*Amino-nitrogen.*—The amino-nitrogen content was found not to vary greatly as the season progressed, as is shown in Table X and Chart IV, Figure B. Such fluctuations as occur are of doubtful significance in relation to winter hardiness of the varieties under study. Gorke (19) was the first to emphasize the precipitation of proteins as a cause for the death of the cell. He showed that this was brought about by increasing the cell sap concentration and its acidity. He found that the precipitation varied with the temperature and hardiness of the plant. Schaffnit (40) ascribed the protection to the splitting of complex proteins into less readily precipitated forms. Later, Schander and Schaffnit (42) restate the hypothesis and relate it to the winter hardiness of cereals. Harvey (23) found an increase in the amino-acid content of hardened cabbage. Newton (34) found a slight increase in the amino-nitrogen content between October and December of all wheat varieties studied. He points out that it appears to be characteristic of all varieties without regard to hardiness. The data cited from other investigators deal with tissues from above-ground parts. In this investigation subterranean parts are involved and, as previously shown, they are not subject to as low temperatures or to the same fluctuations of temperature as the parts above ground. It appears that the same phenomena do not occur under the environment provided by the soil.

The organic nitrogen, which is soluble in alcohol, fluctuates somewhat. However, there is no indication that appreciable amounts of it change to the amino form as a result of low temperature prevailing during the winter months. Thus, this determination does not give a quantitative difference between varieties examined.

The extreme precautions taken by Schander and Schaffnit to prevent any changes in the cell proteins due to temperature were not possible in the preparation of the material for this study. However, with the data at hand, it seems doubtful whether protein splitting exists, if at all, in sufficient degree to function as a means of protection against winterkilling of alfalfa roots. It is also recognized that the alfalfa root is not subjected to wide fluctuations in temperature, especially when covered with snow.

TABLE X

EFFECT OF ENVIRONMENT UPON SOLUBLE NITROGEN IN ALFALFA ROOTS

| Date | Kansas | | | | Grimm | | | |
|---|---|---|---|---|---|---|---|---|
| | Alcohol soluble nitrogen dry weight basis | Amino-nitrogen dry weight basis | Total organic nitrogen dry weight basis | Ratio of amino-nitrogen to total organic nitrogen | Alcohol soluble nitrogen dry weight basis | Amino-nitrogen dry weight basis | Total organic nitrogen dry weight basis | Ratio of amino-nitrogen to total organic nitrogen |
| | Per cent | Per cent | Per cent | | Per cent | Per cent | Per cent | |
| 10/19/22 | 0.72 | 0.39 | ... | .... | 0.76 | 0.45 | ... | .... |
| 12/26/22 | 0.87 | 0.66 | ... | .... | 1.08 | 0.72 | ... | .... |
| 4/23/23 | 0.77 | 0.62 | ... | .... | 1.02 | 0.67 | ... | .... |
| 5/ 5/23 | 0.45 | 0.52 | ... | .... | 0.37 | 0.47 | ... | .... |
| 7/26/23 | 0.63 | 0.41 | ... | .... | 0.73 | 0.40 | ... | .... |
| 8/ 7/23 | 0.84 | 0.51 | ... | .... | 0.86 | 0.47 | ... | .... |
| 10/30/23 | 0.71 | 0.40 | ... | .... | 0.85 | 0.41 | ... | .... |
| 11/10/23 | 0.88 | 0.44 | 2.93 | 0.150 | 0.68 | 0.46 | 2.48 | 0.185 |
| 12/21/23 | 0.48 | 0.43 | 2.84 | 0.151 | 0.71 | 0.54 | 2.68 | 0.201 |
| 1/23/24 | 0.46 | 0.31 | 2.34 | 0.134 | 0.61 | 0.37 | 2.82 | 0.131 |
| 2/ 4/24 | 0.53 | 0.38 | 3.18 | 0.119 | 0.71 | 0.50 | 2.49 | 0.201 |
| 3/13/24 | 0.63 | 0.43 | 2.54 | 0.169 | 0.94 | 0.43 | 3.21 | 0.134 |
| 3/24/24 | 0.75 | 0.46 | 2.99 | 0.154 | 0.88 | 0.43 | 3.10 | 0.139 |
| 4/ 2/24 | 0.73 | 0.39 | 2.95 | 0.132 | 0.94 | 0.45 | 3.09 | 0.146 |
| 4/21/24 | 0.87 | 0.57 | ... | .... | 0.76 | 0.49 | ... | .... |
| 5/17/24 | ... | 0.33 | ... | .... | ... | 0.41 | ... | .... |
| 2/ 7/25 | 0.65 | 0.48 | 2.69 | 0.178 | 0.66 | 0.50 | 2.62 | 0.191 |

## SUMMARY

1. A study has been made of the relative winter hardiness of Grimm and of common Kansas alfalfa. Several available methods have been used with the hope of ascertaining ready means for distinguishing hardy and non-hardy types.

2. Alfalfa plants have no autonomous rest period. The plants came into active growth within 3 days when brought into the greenhouse during every month in which growth is not possible in the field. The plants are forced into inactivity by unfavorable environmental conditions.

3. The varieties under study have a critical period during the early spring interval when the snow disappears and the soil thaws.

4. Resistance to cold increases in the roots of both varieties as winter approaches and disappears with the approach of spring.

5. Temperatures taken at the surface of the soil beneath the snow indicate that snow provides ample protection for alfalfa plants against killing by low temperatures.

6. Freezing injury results in the destruction of the central part of the root and leaves it susceptible to infection by decay organisms. The crown buds are more hardy than the root tissues immediately below them.

7. The thermo-electric method has been used in determining the freezing points of root tissues. It is a rapid method of making determinations but, in general, was not reliable for differentiating between hardy and nonhardy varieties.

8. No absolute correlation exists between the degree of freezing point depression and resistance to killing by freezing.

9. Sap was expressed from the roots and physical determinations were made upon it. The results are summarized here:

a. The quantity of press juice obtained from 100 grams of material at the respective pressures bears no apparent relation to the hardiness of varieties.

b. The total solids in the sap are correlated with total dry matter in the root tissue, but apparently are not correlated with hardiness.

c. Viscosity is related to total solids in the sap but apparently not to hardiness.

10. The alfalfa root undoubtedly functions as a storage structure for carbohydrate and possibly for small amounts of protein reserves.

11. There is a transformation of starch into sugar during the late autumn and early winter and apparently a partial reconversion in early spring, in accordance with the principle of Fischer.

12. It has been found that the hardy variety has more sugar, expressed in terms of total carbohydrates, than the less hardy variety. Accepting the theory that sugar acts as a protective colloid, the hardy variety appears to have a point of advantage.

13. Pentosans have been determined on samples collected during one year, with the result that no apparent quantitative relation exists between pentosan content and hardiness.

14. The amino-nitrogen fluctuations do not indicate that protein cleavage is of importance in the protection of proteins against precipitation.

15. As positive measures of the differences between the varieties under study, the freezing of potted plants or roots removed from the soil has been found to be the most practical and reliable method. Undoubtedly a set of standards could be constructed, based upon the degrees of root injury, which would provide a practical basis for classifying alfalfa strains or varieties into hardiness groups.

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